International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.11 No.07, pp 134-141, 2018

A Study on A Local *Bacillus thuringiensis* SP7 To Control Mosquito Larvae of *Aedes aegypti*

Ahmad Faisal Nasution[1], Dwi Suryanto[1]*, Ameilia Zuliyanti Siregar[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan-20155, Indonesia
[2]Department of Agrotechnology, Faculty of Agriculture, Universitas Sumatera Utara, Medan-20155, Indonesia

Abstract : *Bacillus thuringiensis*(Bt) is a gram-positive bacteria, spore-forming, and producing crystal proteinsused as bioinsecticides. The purpose of the study was to know potential of local isolates of Bt in controlling mosquito larva of *Aedes aegypti*.Isolation of Bt was conducted from soil samples of Tongkoh Forest, Brastagi, North Sumatra, Indonesia. Bt spore and crystal protein were observed using compound light microscope and further using scanning electron microscope. Two suspected isolates were choosen based on their morphological and biochemical characteristic. Further identification was done using their 16S rRNA gene. To know Bt growth pattern in inexpensive C and N-source, Bt isolate was grown in culture media with molasses and urea as C and N sources. To assay on Bt isolate to control mosquito, larva instar 3 of mosquito were put in 50 ml of test media of concentration of 10, 20, 30, 40, and 50% of Bt culture in plastic cup.Observation was done after 24 hrs. Two isolates SP7 and SP15 showed to have morphological and biochemical characteristicsimilar tothat of Bt. Identification based on 16S rRNA gene sequence showed that SP7 and SP15 were closed to *B. thuringiensis* strain MCCC 1A00395 with similarity of 99%. Since two Bt isolates seemed to be similar to *B. thuringiensis* strain MCCC 1A00395, SP7 was choosen for further study. SP7 reached its maximum growth at 30 hrsof incubationtime with cell number of 17.01 log CFU/ml in culture media with molasses and urea as C and N sources. It showed to kill up to 97.5% mosquito larvae atconcentration of 50% bacterial culture.
Keywords : *Aedes aegypti*, *Bacillus thuringiensis*, bacterial cell growth, crystal protein.

Introduction

Malaria, dengue, and several life-treathening diseases was transmitted by mosquitoes. Dengue is a disease transmitted to human through the bite of mosquito *Aedes*. The disease is common in tropical and sub-tropical regions. Data from around the world shows that Asia ranks as the first in the number of dengue sufferers, and Indonesia occupies the highest dengue fever case in Southeast Asia each year[1].Efforts to overcome and preventthe disease through mosquitoe control have been done.

DOI= http://dx.doi.org/10.20902/IJCTR.2018.110716

Chemical insecticides is widely used but the use of insecticides is very unfavorable since it can be harmful to humans and cause environmental pollution and insect resistance[2]. One control considered as safe is to use bioinsecticides. One bacteria *Bacillus thuringiensis*(Bt) is well-known for this purpose[3].

The use of Bt as bioinsecticide has been widely applied to control mosquitoe larva and agricultural insect pests as well. This bacteria kills various mosquito larvae such as larvae of *Aedes*, *Culex*, and *Anopheles*, but no harm for non-target organisms.Special protein is produced by Bt especially toxic to larve of lepidopteran, coleopteran, and dipteran larvae. This protein is a protoxin unless is lysed by alkaline protease in gut of insects[4,5].

Various strains ofBt as bioinsecticide have already been in market. However, study in effectiveness of new isolate to control insect larvae is still on-going, as there are still many disease vector and pest that can not be controlled using existing insecticides[5].Therefore, this study was conducted to assaya newly local Btwhich might be used as bioinsecticide against *A. aegypti* mosquitoe larvae.

Materials and Methods

Soil Sampling andIsolation of Bt from Soil Samples

Soil samples were takenfromTongkohForest, North Sumatra, Indonesia. A 100 g of 2-10 cm depthsoil was collected aseptically using a shovelfrom 3 randomly sites. Samples were put in a sterile plastic bag, and immediately brought to ourlaboratory.

To isolate Bt, modified method by Travers *et al*[6]was utilised. Soil samples of the 3 sites were thoroughly mixed. A 25 g of soil sample wasput into 250 ml of 0.25 M sodium acetate buffer (pH 6.8),shaken strongly for 15 min and heated at 80°C for 30 minutes in waterbath. Suspension was serial diluted in 0.25 M sodium acetate pH 6.8. Diluted suspension was spread in Luria Bertani (LB) agar and incubated at 30°C for 48hrs.Selected colony appeared to have similar morphological and colony colour to Bt was transferred to LB agar and incubated at 30°C for 4-5 days for sporulation[7,8].

Cell and Crystal Morphology

Morphology of bacterial colony was observed including colony shape, elevation, edge, and colour. Cell shape was observed using compound light microscope. Gram staining andbiochemical test was conducted to bacterial cell. To observe crystal morphology, Coomassie Brilliant Blue (CBB) staining was deployed to late culture. Isolates defined as Bt indicated by its crystal proteinwasstored on LB agarfor further study.

Scanning Electron Microscope (SEM)

SEM observation were conducted at Zoological Laboratory, Research Center of Biology,Indonesian Institute of Scicence, Cibinong, West Java, Indonesia. Bt isolate wascultured on LB medium incubated in incubator shakerat 30°Cfor 3 days. Bt culture was spinned at 10.000xg for 10 minutes.Supernatant was discarded and added with 2% coccodylate buffer, then centrifuged again. The fixation solution was discarded. Add coccodylate centrifuged plus 1% tetratoxide, soaked for one hour with this solution. Centrifuged back, tetraoxide solution was discarded and 70%, 80%, 90% and ethanol absolute ethanol were added. The re-centrifuged solution was discarded and added butanol and then suspension was made in butanol. The slip cover piece was frozen and made a suspension review on the slip cover and then dried with freeze-dryer, then coated with gold on vacuum dryer.Observed with JSM-5310LVscanning electron microscope (Japan)[9].

Identification Bt Isolate Based on 16S rRNA Gene Sequence

Bt isolate was cultured in LB agar for 24 hrs.One loop of Bt culture was picked and suspended with sterilled water in microtube. Cell suspension was frozen at -10°C and heated at 90°C for 10 minutes. This was donefor 5 times. Broken cell wasspinned at 10.000xg for 10 minutes. Supernatant contained bacterial DNA was stored at 4°C for further study. DNA in supernatant was amplified using a PCR machine (Sensoquest Labcyler).Amplification of the 16S rRNA gene was conducted using primer 63f (5 '- CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5 '- GGG CGG WGT GTA CAA GGC-3'), which is a universal primer for various strains bacteria[10].PCR reaction was run in mixture of 2 µl DNA template; 12.5 Master Mix 2x

GoTaqGreen; 1 µl (10 pmol) of each primary; 8.5 µl Nuclease Free Water. PCR machine was run at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealingat 55°C for 30 seconds, elongation at 72°C for 1 minute,and post PCR at 72°C for 5 minutes. The reation was conducted for 40 cycles.

The PCR result were visualized on 1% agarose gel (1 g agarose in 100 ml TAE 1X) using minigel electrophoresis. Electrophoresis was performed at 80 volts of 400 mA for 60 minutes. The amplified DNA was visualized with UV-transluminator.

The amplified DNA was purified and commercially sequenced to determine the sequence of its DNA bases. The sequence data was compared tosimilar data in GenBank from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov), using the Local Local Alignment Search Tool (BLAST) program.

Bt Growthon Molasses Urea Medium

Since SP7 and SP15 were actually similar only one isolate of Bt SP7 was choosen for further study. A 2.5 ml of Bt culture (OD_{600}≈0,5) was inoculated in molasses urea salt (MUS) broth composed of1.0 g/l CaCO3; 0.03 g/l MgSO4.7H2O; 0.02 g/l MnSO4.7H2O; 0.02 g/l ZnSO4.7H2O; and 0.02 g/l FeSO4.7H2O. For appropriate growth, 20% molasses (w/v) and 3% urea (w/v) was used [11,12]. Bt cell number was counted using plate count agar methodfor every 6 hrs.

Assay of Bt to Control *A. aegypti* Larvae

Larvae of instar 3 of *A. aegypti* was obtain from Balai Teknik Kesehatan Lingkungan dan Pengendalian Penyakit Kelas I, Medan, North Sumatra, Indonesia.Bt culture of MUS previous culturedwaspoured into plastic cups at concentration of 10, 20, 30, 40, and 50% of 50 ml of well-water test medium with 10 larvae of instar 3 of *A.aegypti* in it. Larvae death was counted in percentage.

Results And Discussions

Isolation of bacteria from soil samples obtained 2potential isolates of SP7 and SP15 (Tabel 1). These 2 isolates showed to have similar morphological and biochemical characteristics to Bt. These two isolates showed to have catalase, motility, hydrogen sulfide production, gelatin hydrolysis, starch hydrolysis but no citrate metabolism which was also observed by Jung *et al*[13], and distincted to other *Bacillus* in which they produced crystal protein.

Table 1:Morphological and biochemical characterization of SP7 and SP15 isolates

Isolates	Colony shape	Colony edge	Colony elevation	Colony color	Gram	Catalase	Motility	Hydrogen sulfide	Gelatin Hydrolysis	Citrate metabolism	Starch Hydrolysis	Crystal
SP 7	Circular	Entire	Flat	Cream	G^+	+	+	yellow	+	-	+	+
SP 15	Circular	Entire	Flat	Cream	G^+	+	+	yellow	+	-	+	+

Notes: += positive reaction;- = negative reaction; G^+= Gram-positive

Observation of Crystal Protein

SP7 and SP15 cells of late culture were stained with CBB dyes. Microscopic observations showed that SP7 and SP15 have spores with solid-shaped crystal protein next to it (Figure 1) which was colored in dark black[14].

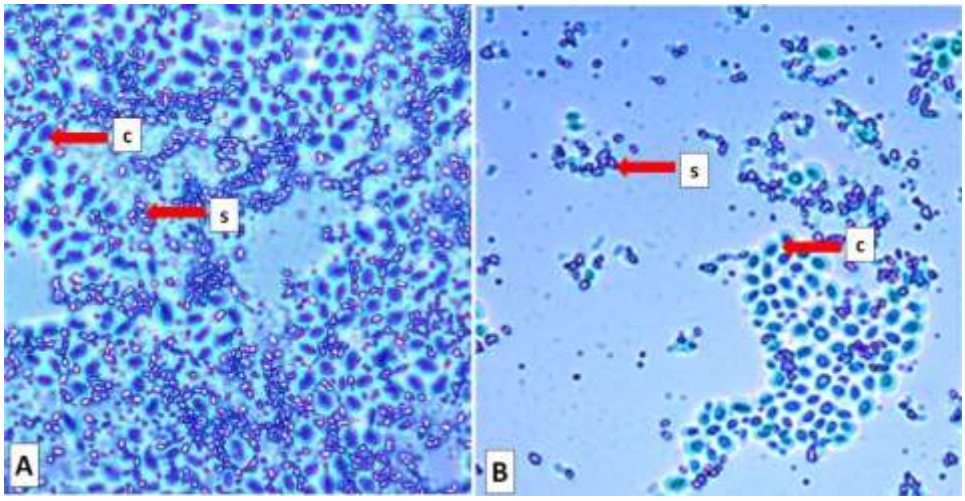

Figure 1.Compound light microscopephotograph of (A) SP7 isolate and (B) SP15 isolate with its spore (s) and crystal protein (c)

Further examination was carried out using SEM techniqueto see more clearly crystal protein shape(Figure 2).The observations using SEM with magnification of 10.000 times showed clearly the shape of spores and crystal proteins. SP7 isolate has spherical crystal protein form, SP15 from observation shows spherical crystal protein form. Other observation showed crystal proteins of spherical shape[15,16,17].

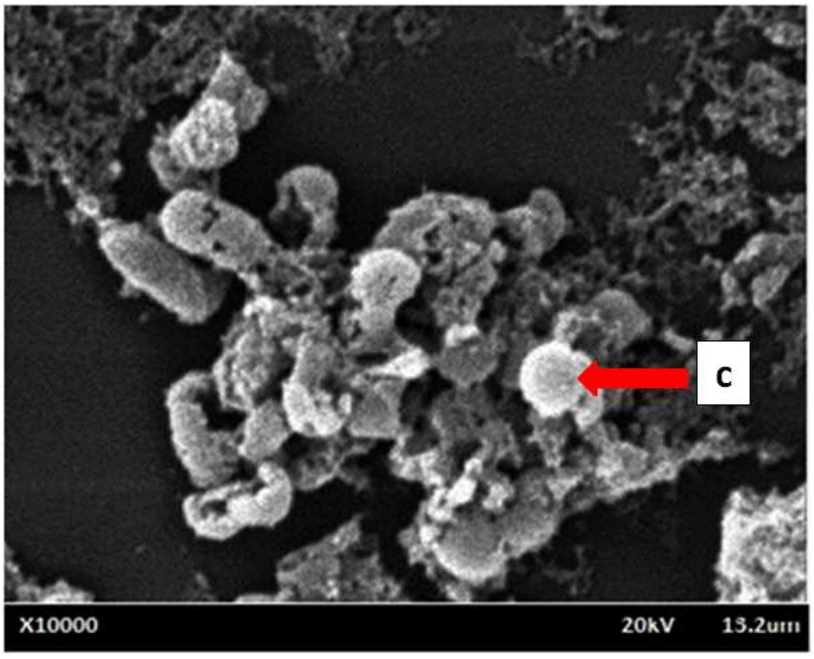

Figure 1. SEM photograph of SP7 isolate and its crystal protein (c)

Identification of Bt Isolates Based on 16S rRNA Gene Sequences

Bt is one of insect pathogenic bacteria. These bacteria belong to the Bacilli class, the Bacillales order, in the Bacillaceae family. These bacteria are aerobes, rodshape, Gram-positive, and spore-forming. They growth in various media. Special characteristics of Bt is that its ability to form crystal proteins along with spore formation[18,19]. Two isolates SP7 and SP15 were identified by using 16S rRNA gene. SP7 and SP15 isolates were closely related to *B. thuringiensis* strain of MCCC 1A00395 strain with 99% similarity (Table 2).

Table 2:Similarity of SP7 and SP15 to *Bacillus thuringiensis* strain MCCC 1A00395based on 16S rRNA gene sequence

Code Isolates	Results of Blast	Similirities	Number of AC
SP7	*Bacillus thuringiensis* strain MCCC 1A00395	99%	KJ812420.1
SP15	*Bacillus thuringiensis* strain MCCC 1A00395	99%	KJ812420.1

Bt Growth in Mollasse Urea Salt Medium

SP7 cell was grown exponentially until 30 hrs of incubation time which reached cell number of 17.01 log CFU/ml, following by its stationary phase (Figure 3). It started to decline after 36 hrs of incubation time. *B. thuringiensis* 47 and Lot2 showed to have exponential phase at 4 hrs and reachedits stationary phase at 36 hrs of incubation time in nutrient broth[20].

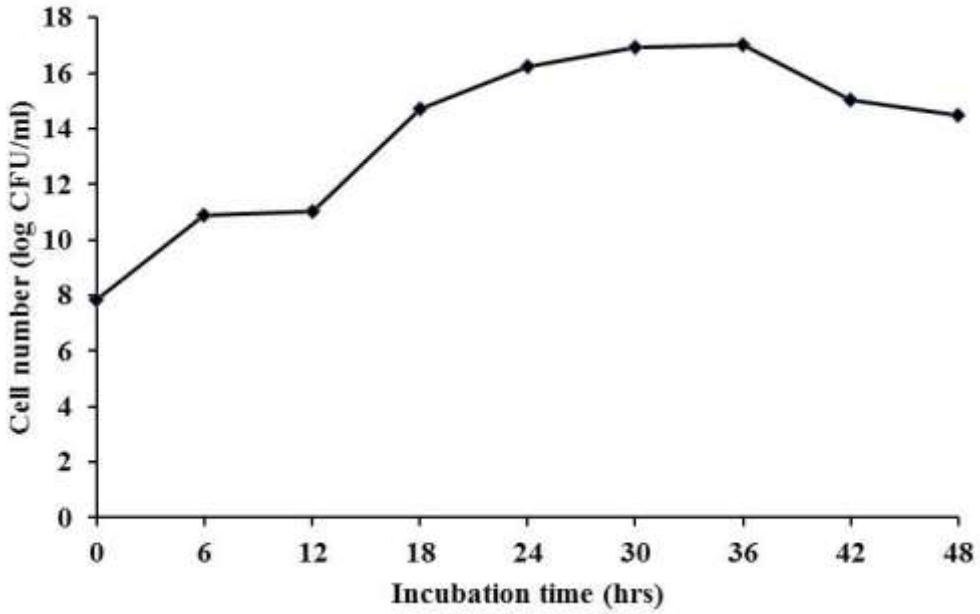

Figure 3.Cell growth profile of SP7 in mollasse urea salt medium

Assay of Bt in Controlling *A. aegypti* Larvae

Assay of Bt in controlling mosquito larvae showed that SP7 was able to kill the larvae up to 97,5%in 24 hrs of SP7 culture concentration of 50%(Figure 4). It was shown that the lower bacterial culture concentration the lower larvae death indicating its toxicity was cencentration dependent.

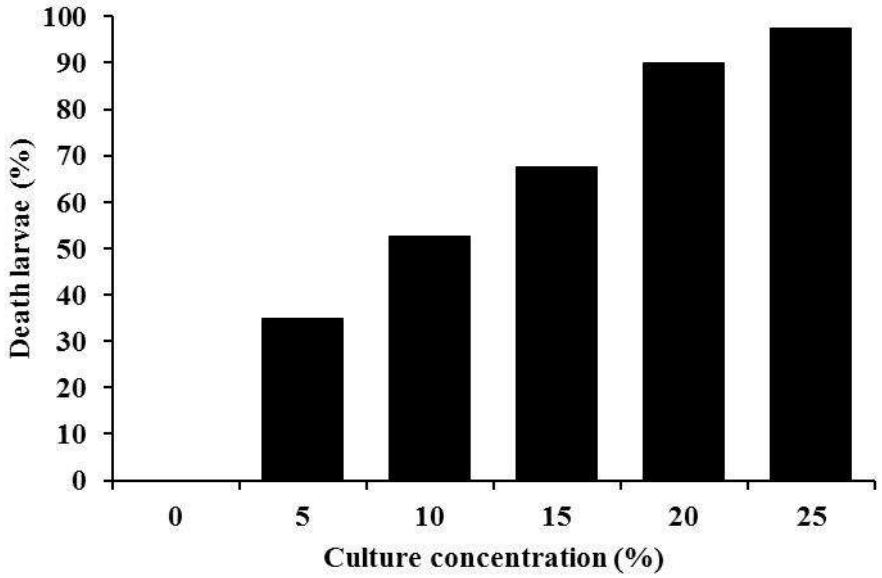

Figure 4. Percentage of mosquito larvae death treated with SP7 in 24 hrs of incubation time

It was said that Bt potential isolate showed to have ability to kill more larvae at concentration below 50%[21]. Another study showed that the percentage of mortality in Bt exposure was 80% over 24 hrswith cell concentration of 4,5 ppm[22]. Gama *et al*[23] showed that *B. thuringiensis* of Madura killed mosquito larvae instar 1up to 88.89%, and high toxicity was found in bacterial density of $1.51x10^8$ cells/ml. Lantang *et al*[24] reported that 19 Bt isolates killed 50% and 3 isolates of which killed more than 80% of mosquito larvae.

Figure 5.Death larvae with blackish gut (arrowed) of *A. agypti* after exposure with SP7.

Mosquito larvae exposed with Bt showed to a damage in its gastrointestinal tract shownby black color (Figure 5). Other indications can be seen from decreasing feeding activity of the larvae[25]. Bt exposure to insect larvae cause insect epithelial organelles to swell and mid intestinal tissue disturbed so that the ability of insect to eat decrease and eventually stopped within a few days[26]. The crystal proteinsingested by the insects are dissolved in the base environment of the target insect intestine andto be activated by an alkaline proteasein insect gut. Activated proteins attach to receptor proteins located on the surface of intestinal epithelial cells resulting in the formation of pores causing the cells undergo lysis, and eventually the insects die[27].

Conclusion

Two isolates SP7 and SP15 were shown tohave similarities to*B. thuringiensis*. The results of crystal protein observation using CBB and SEM technique knew that the crystal proteinshape was spherical. SP7 and SP15 were closely related to *Bacillus thuringiensis* MCCC 1A00395 with 99% similarity. Molasse urea salt medium could be used as growth media of Bt. SP7 showed its maximum growth at 30 hrs of incubation time with cell number of 17.01 log CFU/ml. Itshowed to kill up to 97,5% *A. aegypti* larvae at concentartion of 50% of Bt culture in 24 hour.

Acknowlegment

We would like to thank to University of Sumatera Utara, Medan, Indonesia for partially supported this study.

References:

1. WHO. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. New Edition. Geneva. World Health Organization. 2009. 3-5.
2. Rahayuningsih M., Syamsu K., Darwis A.A., and Purnawati R. Penggandaan Skala Produksi Bioninsektisida *Bacillus thuringiensis* var. *israelensis* Untuk Membasmi Jentik Nyamuk *Aedes aegypti*. 2007. 12(2): 123-130.
3. Aksoy H.M., Saruhan I., Akca I., Onder H., Ozturk M., and Aker O. Isolation and Characterization of *Bacillus thuringiensis* Isolated from Soil and their Possible Impact on *Culex pipiens* Larvae. *Egyptian Journal of Biological Pest Control*. 2015. 25(2): 439-444.
4. Broderick N.A., Raffa K.F.,and Handelsman J. Midgut Bacteria Required for *Bacillus thuringiensis* Insecticidal Activity. *PNAS*. 2006. 103: 15196-15199.
5. Purnawati R., Sunarti C.T., Syamsu K., and Rahayuningsih M. Characterization of Novel *Bacillus thuringiensis* Isolated from *Attacus atlas* and Its Growth Kinetics in The Cultivation Media of Tofu Whey for Bioinsectiside Production. *Journal of Biology, Agriculture and Healthcare*. 2014. 4(16): 33-39.
6. Travers R.S., Martin P.A., and Reichelderfer C.F.. Selective Process for Effecient Isolation of Soil *Bacillus* spp. *Appl. Environ. Microbiol*. 1987. 53: 1263-1266.
7. Moazamian E., Bahador, N., Rasouli M., and Azarpira N. Diversity, Identification and Biotyping of *Bacillus thuringiensis* Strains from Soil Samples in Iran. *Nature Environment and Pollution Technology An International Quarterly Scientific Journal*. 2016. 15(3): 947-950.
8. Senfi F., Safaralizadeh M.H., Safavi S.A., and Aramideh S. Isolation and Characterization of Native *Bacillus thuringiensis* Strains from Apple Prchads at Urma, Iran and their Toxicity to Leptidoptera Pests, *Ephestia kuehniella* Zeller and *Pieric brassicae* L. *Egyptian Journal of Biological Pest Control*. 2012. 22(1): 33-37.
9. Goldstein J., Newburry D.E., Echlin P., Joy D.C., Romig Jr., A.D., Lyman CE., Fiori C., and Lifshin E. Scanning Electron Microscopy and X-Ray Microanalysis: A Text for Biologist, Material Scientist, and Cytologists. 2nd ed. Plemun Press. New York. 1992, 820 p.
10. Marchesi R. J., Sato T., Weightman J.A., Martin A.T., Fry C.J., Hiom J.S., and Wade G.W. Design and Evaluation of Useful Bacterium-Spesific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA. *Applied and Environmental. Microbiology*. 1998. 64(2): 795-799.
11. Nasrah S.N. Viabilitas dan Kerap *Bacillus* sp. BK17 dan *Enterobacter* sp. BK15 pada Sumber Karbon dan Nitrogen yang Berbeda. *Skripsi Biologi USU*: Medan. 2012.
12. Fifendy M., Eldini and Irdawati. Pengaruh Pemanfaatan Molase Terhadap Jumlah Mikroba dan Ketebalan Nata pada Teh Kombucha.*Prosiding Semirata FMIPA Universitas Lampung*. 2013. 67-72.
13. Jung, Chul-Yong, Kim S.UK., Son K.H., Lee H.H., and Bok S.H. Characterization of *Bacillus thuringiensis* Strain BT-14 Having Insecticidal Activity Against *Plutella xylostella*. *Journal of Microbiology and Biotechnology*. 1994. 4(4): 322-326.
14. Chai P.F., Rathinam X., Marimuthu K., Solayyapan M., and Subramaniam S. Soil Enrichment and Dominant Occurrence of *Bacillus thuringiensis* subsp. *israelensis* in a Garden Sprayed with *Bacillus thuringiensis* Based Biopesticide. *Malaysian Journal of Microbiology*. 2014. 10(4): 255-259.

15. Ahmed A.H., Ali G. Salah., and Abdul-Rouf M. Usama. Isolation, Characterization and Molecular Identification of *Bacillus thuringiensis* Alex-13 Isolated fron Egypt Against *Spodoptera littoralis*. *International Jouranl of Microbiology and Allied Science*. 2015. 2(2): 34-44.
16. Sun S, Fan J, Cheng Z, Caiy Y, Li G, dan Pang Y. The Effect of Gamma Strerilization on The Insecticidal Toxicity of Engineered and Conventional *Bacillus thuringiensis* Starins. *Biological and Microbial Contro*l. 2018. 106 (1): 36-42.
17. Silva N.D., Guideli A.M., Abreu I.L.D., Davolos C.C., Polanczyk R.A., dan Lemos M.V.F. Characterization and Selection of *Bacillus thuringiensis* Isolates Effective Against *Sitophilus oryzae*. *Sci. Agric. (Piracicaba, Braz)*. 2010. 67(4): 472-478.
18. Ciccarelli F.D., Doerks T., Von-Mering C., Creevey C.J., Snel B., and Bork P. Toward Automatic Reconstruction of A High Resolved Tree of Life. *Science*. 2006. 311: 1283-1287.
19. Poopathi, S and Archana, B. Optimization of Medium Composition for The Production of Mosquitocidal Toxins from *Bacillus thuringiensis* subsp. *israelensis*. *Indian Journal of Experimental Biology*. 2012. 50(1): 65-71.
20. Khudra A.I. Isolasi Bakteri *Bacillus thuringiensis* dari Tanah dan Pengujian Toksisitasnya Terhadap Ulat Grayak (*Spodoptera litura*). *Skripsi IPB*: Bogor. 2011.
21. Yamamoto T., Lizuka T.and Aronson J.N. Mosquitocidal Protein of *Bacillus thuringiensis* var *israelensis*: Identification and Partial Isolation of The Protein. Current. *Microbial*. 1983. 9: 279-284.
22. Yunus R. and Satoto T.B.T. Efikasi *Bacillus thuringiensis* yang Ditumbuhakan Pada Media Air Cucian Beras Mekongga Terhadap Larva *Aedes agypti* Strain Kendari. *Vektora*. 2017. 9(1): 9-16.
23. Gama Z.P., Yanuwiadi B. and Kurniati T.H. Strategi Pemberantasan Nyamuk Aman Lingkungan: Potensi *Bacillus thuringiensis* Isolat Madura Sebagai Musuh Alami Nyamuk *Aedes agypti*. *Jurnal Pembangunan dan Alam Lestari*. 2010. 1(1): 1-10.
24. Lantang D. and Runtobi Y. Dirki. Karakterisasi Bakteri *Bacillus thuringiensis* Asal Hutan Lindung Kampus Uncen Jayapura, serta Deteksi Toksisitasnya Terhadap Larva Nyamuk *Anopheles*. *Jurnal Biologi, Papua*. 2012. 4(1): 19-24.
25. Wibowo, C.I.Efektifitas *Bacillus thuringiensis* dalam Pengendalian Larva Nyamuk *Anopheles* sp. *Biosfera*. 2017. 34(1): 39-46.
26. Chilcott C.N., Knowles B.H., Ellasr D.J., and Drobniewski F.A. Mechanisms of Action of *Bacillus thuringiensis israelensis* Parasporal Body. *Bacterial Control of Mosquitoes and Blackflies*. 1990. 45-46.
27. Wabiko H., Raymond K.C., and Bullajr L.A. *Bacillus thuringiensis* Entomocidal Protoxin Gene Sequence and Gene Product Analysis. *DNA*. 1986. 5: 305-314.

*****